Application of fluorescence polarization to the steady-state enzyme kinetic analysis of calpain II

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Abstract This paper presents the application of fluorescence polarization to the determination of dissociation constants for competitive inhibitors that bind to enzymes. This steady-state enzyme kinetic study measures the inhibition of the conversion of a fluorescently tagged substrate to a lower molecular weight fluorescent product by calpain II. It relies on the measurement of a parameter proportional to velocity, which is sufficient for this type of analysis. The strengths and limitations of the method are discussed. Inhibition constants for filamin and spectrin determined by this method are 125 nM and 13 nM respectively.

Key words: Calpain; Fluorescence polarization; Enzyme assay; Spectrin; Fodrin

1. Introduction

Fluorescence polarization (FP) is used increasingly to measure dissociation constants between receptors and ligands. The theory behind FP was first described in 1926 [1]. Plane polarized light is used to excite a fluorescent molecule such as fluorescein, and after a limited lifetime (4 ns for fluorescein), light is emitted from the molecule and detected by a photomultiplier tube in planes parallel and perpendicular to the polarized light source. If, during the lifetime of this excited state, the molecule tumbles significantly, the light is depolarized so that intensity is observed in both planes. Thus, a large polarization value (500 milli-polarization units being the theoretical maximum) is associated with a slowly tumbling molecule, and slow tumbling is associated with large molecular size, in addition to high solvent viscosity and low temperature [2–4].

FP has been used to detect enzymatic cleavage of high molecular weight, fluorescently tagged proteins to produce faster tumbling fluorescently tagged peptides. These studies typically measure empirical enzyme activity levels [4–8], although in one case a Lineweaver-Burk analysis was carried out based on percent changes in polarization as a function of time [9]. A limitation of the latter type of analysis is that the polarization change for complete conversion of substrate to product is needed, and it is not always possible to obtain this number. An enzyme kinetic study is reported here, using calpain II as a model system to demonstrate an approach that allows for quantitative steady-state initial velocity studies to be carried out using FP, allowing for the determination of Michaelis constants for substrates, and inhibition constants for inhibitors.

Calpain is a calcium-activated protease that cleaves the cytoskeletal proteins spectrin, fodrin and filamin, in addition to numerous other substrates [10]. Spectrin and fodrin are closely related proteins comprised of two α and two β subunits, each of approximate molecular weight 240 kDa, while filamin is a dimer comprised of two 240 kDa subunits [11,12]. All three proteins bind to actin [11,13–15]. Autolysis of the large (80 kDa) subunit of calpain II results in the rapid loss of enzyme activity in in vitro assays [16], so rates must be measured in the first minute or so of reaction to get reliable initial velocities.

2. Materials and methods

2.1. Reagents for calpain kinetic experiments

Leupeptin, chicken gizzard filamin, and the 80 kDa subunit of type II calpain from rabbit skeletal muscle were obtained from Sigma. Calpain stock solutions were prepared in PBS buffer, pH 7.2, comprised of 10 mM phosphate and 150 mM NaCl. Filamin was labeled with approximately 1–2 FITC molecules per filamin molecule using the Sigma FluorTag kit and the manufacturer's suggested protocol. A 1 mg/ml stock solution in 10 mM PBS was stored in the dark at 4°C and used within 2 weeks of preparation. Human erythrocyte spectrin from Sigma was dialyzed against 10 mM PBS and 1 mM DTT to remove residual phenylmethylsulfonyl fluoride. All calpain steady-state kinetic experiments were performed in a 1 ml volume at 23°C (ambient) with a Beacon (PanVera) instrument. The reaction buffer contained: 50 mM Tris, pH 7.3, 5 mM L-cysteine, 4 mM $CaCl_2$, 2.5 mM β-mercaptoethanol and 4% dimethyl sulfoxide, except in the leupeptin inhibition study[1], where buffer contained: 110 mM imidazole, pH 7.3, 5 mM L-cysteine, 5 mM $CaCl_2$, 2.5 mM β-mercaptoethanol, 2% dimethyl sulfoxide and 2% methanol.

2.2. Steady-state kinetics and data analysis

After blanking on buffer only, the initial polarization was read for buffer and FITC-filamin, enzyme was added and kinetic readings were taken every 15 s. FITC-filamin was varied around its K_m. For competition experiments, varied concentrations of non-FITC labeled competitor ligand were also included. Initial velocities were taken in the first 60 s, before the enzyme lost activity from calcium activated autolysis. The ΔP_t value is obtained by subtracting the initial polarization value from that after 1 min. The initial velocity that is measured is the change in polarization per unit time, $\Delta P_t/\Delta t$, and the actual rate required for steady-state enzyme kinetic analysis is the change in substrate concentration per unit time, $\Delta A_t/\Delta t$. The fraction of FITC labeled substrate consumed is approximated by:

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Abbreviations: FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; FP, fluorescence polarization; Tris, tris(hydroxymethyl)aminomethane

[1] The assay with leupeptin had to be carried out with an imidazole buffer, rather than Tris, as Tris forms a Schiff base with the aldehyde on leupeptin. As a result of Schiff base formation, an anomalously high K_i of 50 ± 15 μM was obtained when Tris was used as buffer. Comparing the K_i of 50 μM in Tris with that of 1.0 μM in imidazole buffer suggests that 98% of the aldehyde is present as the imine in 50 mM Tris, pH 7.3.

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$$\chi_A = \frac{P_o - P_t}{P_o - P_f} = \frac{\Delta P_t}{\Delta P_c} \quad (1)$$

where P_o is the polarization of FITC labeled substrate before adding enzyme, P_t is the polarization after reaction for time t, and P_f is the polarization after FITC labeled substrate has been completely converted to FITC labeled product[2]. So, the fraction of substrate consumed at time t is the ratio of the polarization change at time t (ΔP_t) to that for the complete conversion of substrate to product (ΔP_c). This is the percent change in polarization discussed in a previous report of FP being used for steady-state enzyme kinetic analysis [9]. In the case where a population of different sized products is produced, as in the proteolysis of a fluorescently tagged protein, this equation is a reasonable approximation if initial rates are measured for a substrate that is minimally labeled with FITC. Under these conditions, initial rates will be dominated by the fastest reaction. For this reason it is essential not only to measure initial rates, but to verify that progress curves are linear. Deviation from linearity may also occur if substrate and product(s) differ significantly in total fluorescence intensity[3]. If progress curves are linear at early time points, then χ_A values obtained from Eq. 1 are reasonable approximations of the fraction of substrate consumed, and can be used in the following analysis. The enzyme initial velocity (v) in units of [concentration]/[time] is obtained from the measured change in polarization (ΔP_t) using:

$$v = \frac{\Delta A_t}{\Delta t} = \frac{\chi_A A_o}{\Delta t} \quad (2)$$

where A_o is the FITC-substrate concentration at the start of the reaction. A determination of χ_A requires a knowledge of P_f (Eq. 1), which cannot be determined unless the FITC-substrate is completely converted to FITC-product. Most of the time, an enzyme is unstable, there is product inhibition, and/or a reaction is not thermodynamically favorable enough to force the complete conversion to products, making a determination of P_f impossible. Since ΔP_c is the same for all FITC-substrate concentrations, an apparent rate can be calculated from Eqs. 1 and 2:

$$v' = [\Delta A_t/\Delta t](\Delta P_c) = [\Delta P_t/\Delta t](A_o) \quad (3)$$

where $v' = (v) \times (\Delta P_c)$, and has the same units of concentration as A, per unit time. Although the steady-state parameters V_{max} and (V_{max}/K_m) obtained using these apparent rates will be ΔP_c-fold larger than their actual values, Michaelis and inhibition constants will be unaffected, so there is no need to determine the ΔP_c correction factor. Thus, the Michaelis constant (K_m) for FITC-filamin binding to calpain (Fig. 1A,B) was obtained from a fit to:

$$v' = \frac{V_{max}' A_o}{K_m + A_o} \quad (4)$$

where A_o is the starting concentration of FITC-filamin and V_{max}' is (V_{max})(ΔP_c). Competitive inhibition constants, K_i, for filamin (Fig. 2A), spectrin (Fig. 2B) and leupeptin (Fig. 2C) were obtained by fitting the data to the equation for competitive inhibition:

$$v' = \frac{V_{max}' A_o}{K_m(1 + I/K_i) + A_o} \quad (5)$$

where I is the concentration of non-FITC labeled inhibitor, and K_i is its competitive inhibition constant. Steady-state kinetic data were fitted using the FORTRAN programs of Cleland [17] which perform non-linear least squares optimization. Data were fitted in log form, since this assumes constant proportional error in v'. Steady-state kinetic analysis requires that FITC-substrate be varied in concentrations around its K_m, but this may be technically difficult if a substrate has a high K_m value, since the higher substrate concentrations in this analysis need to be 3–5 times K_m, and this much FITC may overload the instrument's photomultiplier tube. If competitive inhibition is expected, then it is acceptable to vary substrate at concentrations far below K_m, since the required slope (V_{max}/K_m) effects will be well defined, and there will be no intercept (V_{max}) effects.

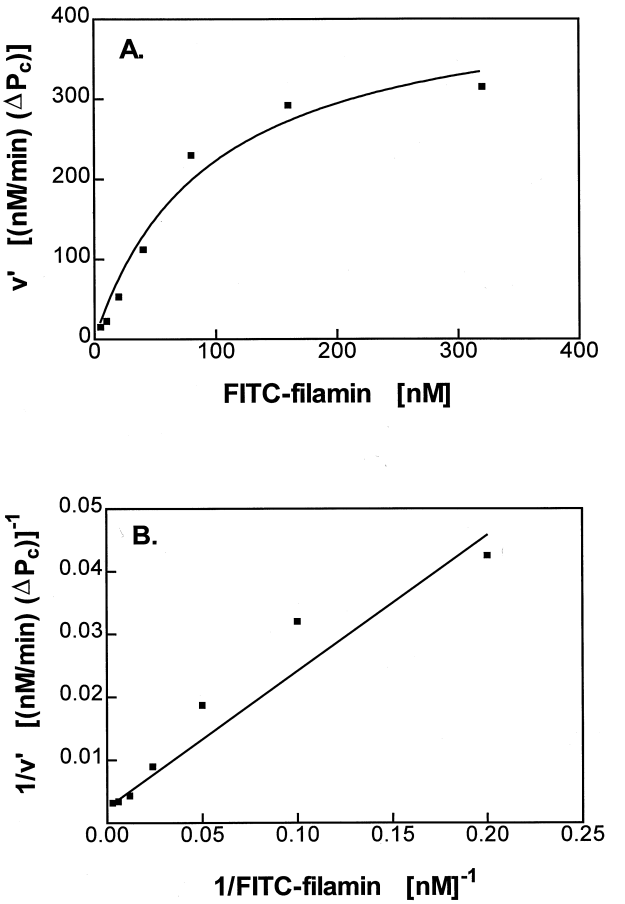

Fig. 1. Steady-state enzyme kinetic initial velocity plots using FITC-filamin as a substrate for calpain II, with velocity v' obtained from changes in polarization as defined in Eq. 3. Saturation of the enzyme is apparent in A, and B shows the linearity of the double reciprocal plot. Calpain was present at 200 ng/ml.

3. Results and discussion

A method for doing steady-state enzyme kinetics using fluorescence polarization is presented, with application to calpain II. Initial rates were obtained in the first 60 s by measuring the change in polarization per unit time. Progress curves were linear for this initial period, before autolysis and other effects (discussed above) caused a slowing of the reaction. This rate is multiplied by the starting FITC-substrate (filamin) concentration to get an apparent rate, v' (Eq. 3). The change in polarization (ΔP_t) over a period of 1 min (Δt) was 4–12 mP[4], which after multiplying by the FITC-filamin substrate concentration (A_o) spanned a 20-fold range of apparent velocities (v'). These apparent rates were then used in the initial velocity

[2] To the extent that total fluorescence intensity ($I_{\parallel} + 2I_{\perp}$) is constant, Eq. 1 applies equally to polarization and anisotropy measurements.

[3] This is in addition to the usual causes of deviation from linearity due to approach to equilibrium, or product inhibition if product accumulates to concentrations close to its K_d.

[4] The sensitivity and precision of this method are best if the measured change in polarization (ΔP_t) is large. But, because of the need for linear progress curves, initial rates were taken here, which were still well within the range of accurate measurement. The accessible dynamic range (some fraction of ΔP_c) will be decreased if the difference in mobility of FITC attached to substrate, compared to that attached to product, is small. This is often the case if there is significant internal/segmental motion of the FITC group attached to the substrate.

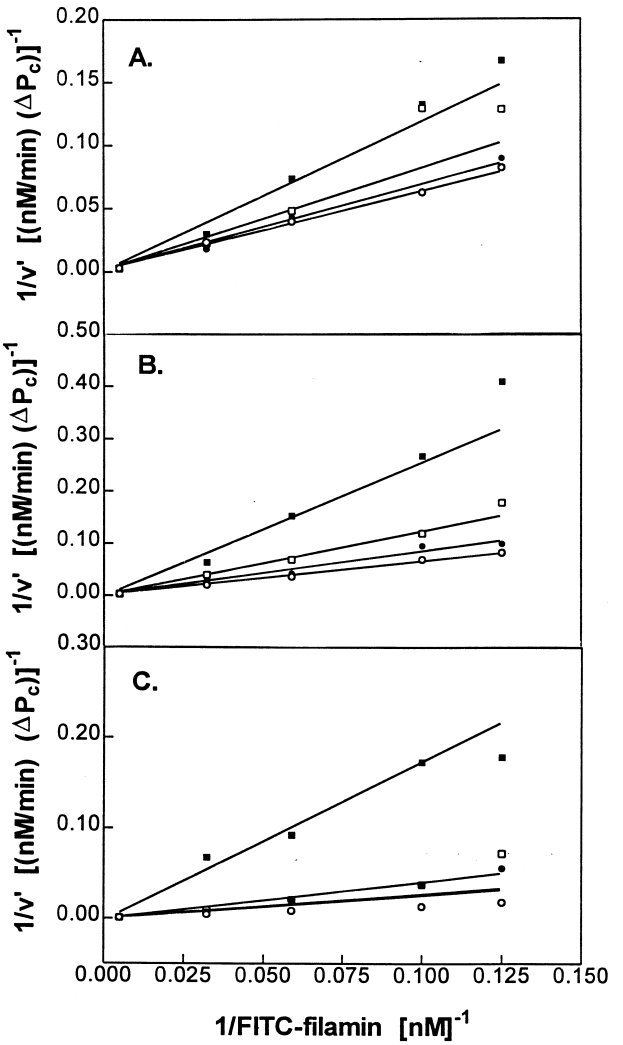

Fig. 2. Steady-state enzyme competitive inhibition curves with FITC-filamin as the varied substrate and each line corresponding to a different inhibitor concentration. Calpain was present at 20 ng/ml. Inhibitor concentrations corresponding to the open circles, closed circles, open squares, and closed squares were: (A) 0, 13, 42, 125 nM with filamin as inhibitor, (B) 0, 4, 13, 42 nM with spectrin as inhibitor and (C) 0, 0.1, 1, 10 μM with leupeptin as inhibitor [18].

study by fitting the data in Fig. 1A (and Fig. 1B) to Eq. 4, giving a K_m for filamin of 98 ± 29 nM. Competitive inhibition studies with varied unlabeled filamin as inhibitor were also done using these apparent rates (v') and fitting the data in Fig. 2A to Eq. 5 to give a K_i of 125 ± 42 nM, very close to its K_m value. The K_i for spectrin was determined in the same manner, and is 13 ± 4 nM (Fig. 2B). This interaction is slightly stronger than that reported between calpain I and fodrin, which had a K_m of 50 nM [13]. Finally, the K_i for the protease inhibitor leupeptin was determined to be 1.0 ± 0.6 μM[1] (Fig. 2C), which is close to the previously reported value of 0.43 μM for calpain II [18].

In summary, FP is a useful tool for steady-state enzyme kinetic analysis, especially for competitive inhibition studies. Although high substrate concentrations may overload the photomultiplier tube, this is not a problem when determining K_i values for competitive inhibitors, as lower substrate concentrations can be used. Of course, substrate must still be kept in significant excess over enzyme. Photomultiplier tube overloading could be avoided with appropriate filters on FP instruments. But, care must be taken to make sure progress curves are linear, since Eq. 1 is an approximation that is valid only if: (1) initial rate is dominated by the production of one fluorescent product (and not a population with different ΔP_cs) and (2) the total fluorescence intensity per species is the same[2]. Linear progress curves are an indication that these two conditions are met, and that an apparent rate at least proportional to the actual rate is being measured. Fortunately, determination of Michaelis and competitive inhibition constants can be done with an observable that is only proportional to initial velocity, so it is not necessary to determine the absolute change in fluorescence for complete conversion of substrate to product (ΔP_c in Eq. 1), which in most cases would be difficult to determine.

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